

## Transgene integration – an analysis in autotransgenic *Labeo rohita* Hamilton (Pisces: Cyprinidae)

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**Abstract** Transgenic *Labeo rohita* founder population was analyzed for the presence of autotransgene having histone 3 promoter and growth hormone (GH) cDNA (LRH3-GHcDNA) or total GH gene (LRH3-GH2.8) by PCR with transgene specific primers. Transgene specific amplification was seen with LRH3-GHcDNA in five out of seven individuals and all three fishes with LRH3-GH2.8, indicating their transgenic nature. Transgene integration was also studied by Southern hybridization of DNA isolated from blood of the transgenic fishes with two different probes (histone 3 promoter and cDNA of *L. rohita*). Autotransgene integration was confirmed in all PCR positive transgenic individuals. The site of integration of the transgene in the genome of the four transgenic fish could be determined by inverse PCR. Two individuals showed integration at the same site whereas in the remaining two individuals the integration sites were different.

**Keywords** *Labeo rohita* · Autotransgene · Transgene integration · Inverse PCR

### Introduction

Use of heterospecies gene constructs in transgenesis faced problems that were related to non-acceptability of such fish for human consumption, increased ecological risks as well as certain ethical issues (Hallerman and Kapuscinski 1995; Pandian 2001). The use of homologous gene sequences (with respect to both the regulatory and structural gene sequences) derived from the same species in an ‘autotransgene’ (Beardmore 1997) construct, is now widely advocated for the development of transgenic fishes. Nam et al. (2001) first reported autotransgenic mud loach, *Misgurnus mizolepis* having a growth hormone (GH) gene and  $\beta$  actin promoter isolated from the same species, which showed higher growth than the wild type. Another important criterion that has emerged for transgene construction is the use of the chromosomal gene sequence as structural gene as it has been shown that the presence of intron in several cases increased transcription efficiency (Brinster et al. 1988; Hir et al. 2003).

Indian major carps (*Labeo rohita*, *Labeo calbasu*, *Catla catla*, *Cirrhinus mrigala*) constitute 75% of the total fish production in India (Reddy et al. 1999). Driven by market preferences, the emphasis has been on selective breeding for enhanced growth in *Labeo rohita*, which resulted in a strain ‘jayanthi’, that showed 18% higher growth in each successive generation (Mahapatra et al. 2000). To obtain further enhancement of growth, transgenic approaches were

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adopted. With “all-fish” transgene construct (pgc  $\beta$ -rGH-IRES-EGFP) having GH cDNA from *L. rohita* attached to a  $\beta$  actin promoter from grass carp (a Chinese carp), the transgenic *L. rohita* showed five to six times higher growth, whereas the transgenic fish with heterospecies transgene construct (pCMV-rGH-IRES-EGFP) showed only four times higher growth than nontransgenic individuals (Venugopal et al. 2004). Rajesh (2004) reported development of transgenic *L. rohita* with ‘autotransgenic’ constructs having histone 3 promoter and the GHcDNA (LRH3-GHcDNA) or total GH structural gene (LRH3GH2.8) with its 3’ regulatory sequences (communicated for publication) which also showed four to five times higher growth than the nontransgenic controls.

To obtain a homozygous transgenic stock, identification and integration of transgene in the putative transgenic individuals is essential before embarking on breeding of such fish. Identification of transgenic fish and the integration of the transgene were confirmed by PCR and Southern hybridization respectively in mud loach (Nam et al. 2001), *L. rohita* (Venugopal et al. 2004) and coho salmon (Devlin et al. 2004). In the present study, ‘autotransgenic’ *L. rohita* obtained by Rajesh (2004) were subjected to PCR and Southern analysis with transgene specific primers and probes confirming the transgenic nature and the integration of the transgene in these fish. In addition, inverse PCR analysis also identified the site of integration of the transgene in the genome of the transgenic fish.

## Materials and methods

DNA was isolated by proteinase K method (Sambrook et al. 1989) from fresh blood, eye, fin, heart, intestine, liver and muscle tissues of fish.

For Southern analysis, 10  $\mu$ g of genomic DNA was digested to completion with restriction enzymes (*Hind*III or *Eco*RV), size fractionated on 0.8% agarose and transferred to Hybond N<sup>+</sup> membrane (Majumdar et al. 1997). Hybridization was carried out at 60 °C in 0.5 M phosphate buffer, (pH 7.5) and SDS with P<sup>32</sup> labeled probe (specific activity 10<sup>8</sup>–10<sup>9</sup> cpm/ $\mu$ g) at a concentration of 1–5 $\times$ 10<sup>6</sup> cpm/ml for 16–18 h. The membrane was washed twice in 0.1 $\times$ SSC and 0.1% SDS at 60 °C for 30 min each and exposed to X-ray film at –70 °C for 2–3 days with intensifying screen.

Hot start PCR, using a thermal cycler (MJ Research, USA) in a final reaction volume of 25  $\mu$ l with genomic DNA (100 ng), Taq polymers (1 unit); nucleotides (100  $\mu$ M), primers (10 pmol) and MgCl<sub>2</sub> (2.5 mM), was done for 35 cycles with denaturation at 95 °C for 25 s, annealing at 55–60 °C for 50 s, extension at 70 °C for 60 s. Final extension was for 5 min at 70 °C and the product obtained was analyzed on a 1% agarose gel.

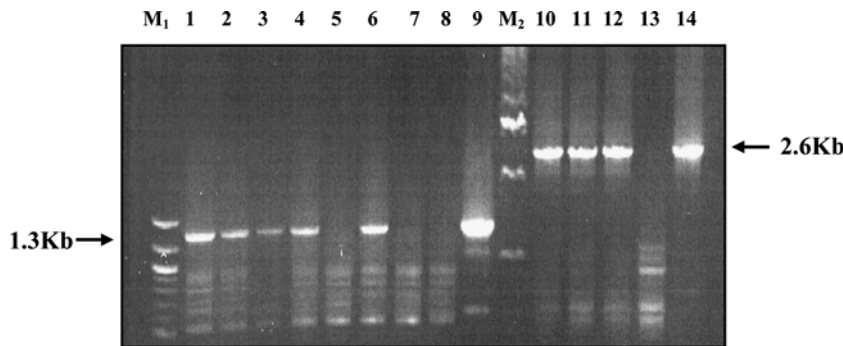
DNA sequencing was done in an automated DNA sequencer (AB1 3700).

## Results and discussion

### Identification of putative transgenic fish by PCR

Autotransgenic founder *L. rohita* population (LRH3-GHcDNA or LRH3-GH2.8 constructs) were obtained through fertilization of eggs with spermatozoa electroporated with the transgene without the plasmid backbone (The details of the electroporation conditions, DNA concentrations and success rates have been sent separately for publication). Out of the several putative transgenic individuals that were obtained with autotransgene, LRH3-GHcDNA and LRH3-GH2.8 constructs only seven from the former and three individuals from the latter group surviving to their adulthood and were used in the present study. DNA isolated from blood samples of individuals were used for PCR amplification with autotransgene specific primers (H3F, 5’-GAGAAGGCCGTCAAA-GCCAAGT-3’ and LR3’UTR, 5’TTTAATTTATG-AGCACTCCCTAA-3’, or LR4ER, 5’-CAAGTCGGCCAGCTTTTCATT-3’). Results indicate that out of all the individuals that were expected to have LRH3-GHcDNA, only (28%) individuals (Fig. 1, lanes 5 and 7) did not possess the transgene. On the other hand, all the individuals that survived from the LRH3-GH2.8 group had the transgene (Fig. 1). This method of identification of transgene has been used in several other fish species (Dunham et al. 1992; Nam et al. 1999) although it does not establish transgene integration, as well as mosaicism of transgene at the level of individual tissue.

DNA isolated from different (eye, fin, heart, intestine, liver, muscle) tissues of an individual with LRH3GHcDNA construct when subjected to PCR



**Fig. 1** Detection of transgene in the putative transgenic *Labeo rohita*. Genomic DNA isolated from individuals is amplified by PCR using autotransgene specific primers reveal that the H3-GHcDNA construct is present in the genomic DNA of the 5 individuals (lanes 1, 2, 3, 4, and 6) out of a total of 7 individuals used in the study. On the other hand all individuals (10, 11, and 12) show amplification in which pLRH3-GH2.8

autotransgene is used for the development of transgenic individuals. The background amplification at the lower side of the gel may be due to internal primer binding sequence specific for H3 or GH. Arrow indicates transgene specific amplification. Lanes 9 and 14 represent plasmid control, lanes 8 and 13 represent nontransgenic fish, M1 and M2 represent molecular weight markers

by two sets of primers (H3F and LR2ER, 5'-AAAGTCGTTAATCATTTCAGCCAG-3'; H3F and LR4ER), amplifications were obtained with both sets of primers from all tissues except the intestine (Fig. 2). Absence of amplification in the intestine with both sets of primers strongly suggests a mosaic nature of the presence of transgene in different tissues. Similar result was reported for the same species by Venugopal et al. (2004).

#### Analysis of transgene integration

The foregoing studies established the presence of transgene. Further studies involving Southern hybridization with two different probes, cDNA of GH and histone 3 promoter of *L. rohita* on the DNA from these transgenic fish helped in analyzing the integration of the transgene.

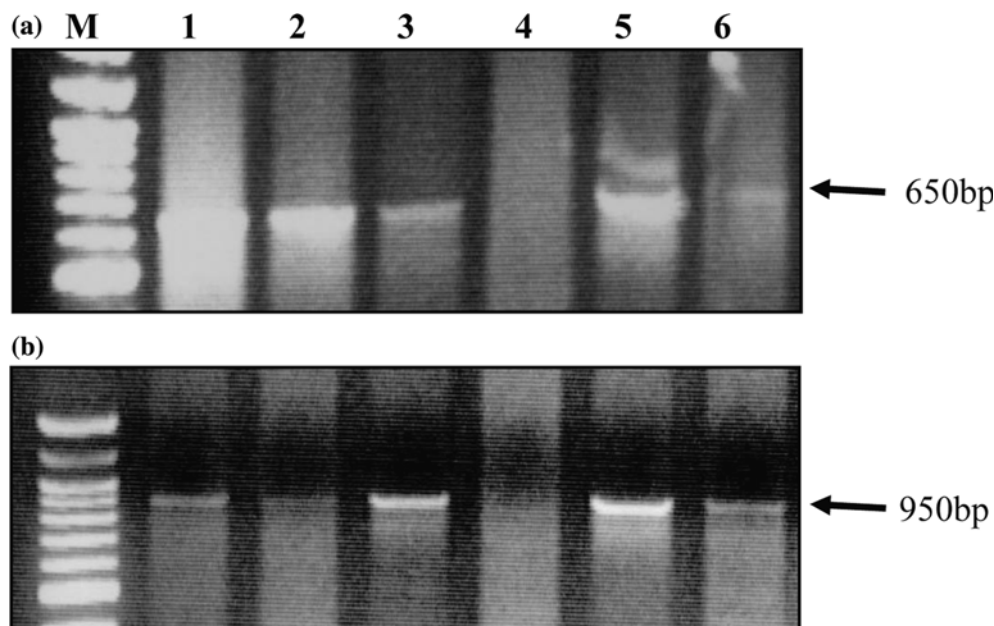
#### Hybridization with growth hormone cDNA probe

The Southern hybridization experiments with GH probe (cDNA) of putative transgenic individuals shows two fragments (8kb and 1kb) that were consistently present in all individuals, after *Hind*III digestion (lanes 1–11 Fig. 3, panel II). These represent the endogenous GH gene, which is also seen as expected in lane 11 (wild type individual) and in lanes 5 and 7 (PCR negative). Lanes 2 and 3 show similar hybridization (5.2, 2 and 0.5 kb, region) patterns and lanes 8–10 show hybridization at 5 and

1.7 kb which are similar in all three individuals having transgene LRH3-GH2.8. The individuals with transgene LRH3-GHcDNA, however, show a difference in the hybridization pattern (lanes 1–7). The same DNA samples when digested with *Eco*RV (Fig. 3, panel II) and hybridized with GH cDNA, the band at 8.5kb region in all individuals represents the endogenous GH gene. Additional hybridization bands are seen in lane 1 at 5, 2.6 and 1.7 kb regions in lane 4 and 6 at 5 and 1.7 kb regions respectively, and in lanes 8–10 at 5, 3.5 and 1.7 kb region respectively. Transgenic fish with LRH3-GH2.8 show similar pattern of hybridization with the same enzyme-probe combinations, whereas individuals with LRH3-GHcDNA show dissimilarities in hybridization patterns with the same enzyme-probe combination. Similar patterns of hybridization may indicate similarity in the integration site, which has earlier been reported in transgenic coho salmon (Devlin et al. 2004). Besides, the transgenic fish do not possess any free copy of autotransgene in our studies which is in contradiction with the result obtained by Venugopal et al. (2004) which may be attributed to the differences in the conditions used in sperm electroporation for transfer of transgene in these two sets of experiments.

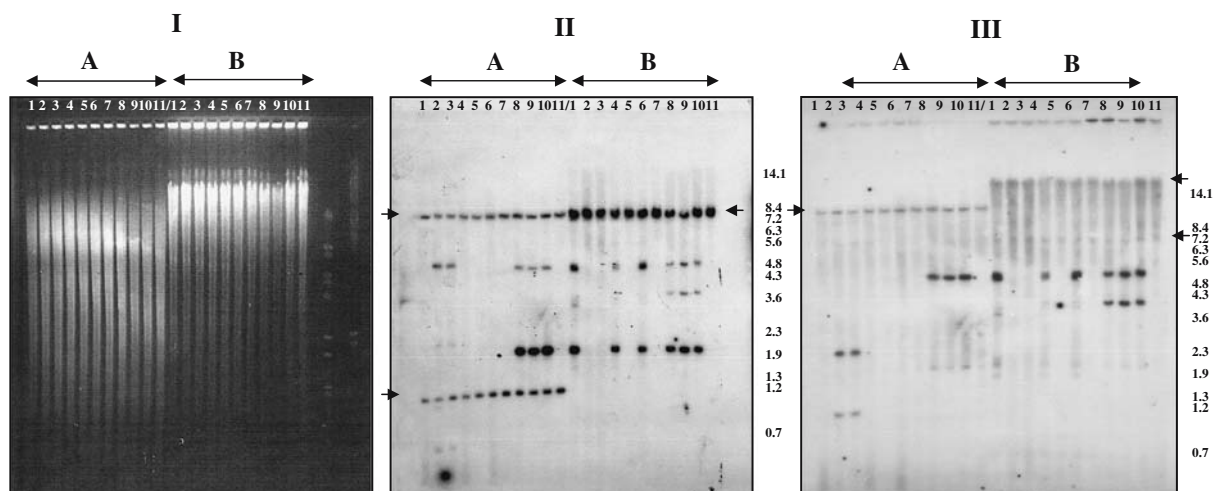
#### Hybridization with histone 3 promoter probe

To further establish the integration of the autotransgene, the same blot was rehybridized with H3 probe (Fig. 3 panel III). Samples digested with *Hind*III



**Fig. 2** *Labeo rohita* showing chimerism with respect to the transgene in different tissues. Two sets of primer are used for transgene specific amplification as shown in the panel a (Primers H3F and LR2ER) and panel b (Primers H3F and

LR4ER). In both the panels all the tissues show amplification except lane 4 which does not show amplification. Lanes 1–6 are eye, fin, heart, intestine, liver, and muscle respectively. M represents molecular weight markers



**Fig. 3** Southern analysis of the transgenic fishes using GH cDNA (panel II) and histone 3 promoter (panel III) probe. A, *Hind*III digestion and B *Eco*RV digestion. Panel I-UV photograph of the digested sample. Lanes 1–7, H3-GHcDNA transgene construct and lanes 8–10, H3-GH2.8 transgene

construct. Lane 11, wild type *Labeo rohita* DNA. Same individuals are reported in the same lanes in both panels. Arrowheads indicate hybridization bands of endogens GH (in panel II) or H3 promoter (panel III)

showed hybridization at 10 kb whereas, the same samples digested with *Eco*RV (Fig. 3, panel III) showed hybridization at 20 and 7.5 kb regions. These bands represent the endogenous histone 3 gene seen

in nontransgenic individuals and in fish that are PCR negative for the autotransgene (lanes 5, 7, 11, Fig. 3, panel III). The samples digested with *Hind*III show hybridization at 2 and 1 kb regions in lanes 2 and 3

and at 5 and 1.7 kb regions in lanes 8 to 10. The same samples when digested with *EcoRV* show a different pattern of hybridization. In this case, lane 1 has three hybridization signals at 5, 2.6 and 1.7 kb regions whereas lanes 4 and 6 show hybridization only at the 5 kb region. In lanes 8–10, two hybridization bands were seen at 5 and 3.5 kb regions. Hence, variations in hybridization are noticed mainly in transgenic fish with LRH3-GHcDNA transgene, whereas transgenic fishes with LRH3-GH2.8 show similar hybridization patterns between the individuals.

The hybridization signals in 8, 1 and 8.5 kb (Fig. 3, panel II) regions are due to the endogenous GH gene and the hybridization signal at 10, 20 and 7 kb (Fig. 3, panel III) are due to the endogenous histone 3 promoter. All the other hybridization bands are due to the integration of the autotransgene having histone 3 promoter and GH gene. All the bands assigned to the autotransgenes are accounted for and it is seen that the endogenous growth hormone and histone genes do not show any overlap in their hybridization patterns. Besides, it is also possible to identify, on the basis of hybridization intensities the bands that correspond to the histone 3 or GH gene portions in the integrated autotransgene. For example, the band at 1.7 kb (in Fig. 3 Panel II) is from the GH gene, whereas the band at 5 kb (Fig. 3 Panel III) is from H3 gene.

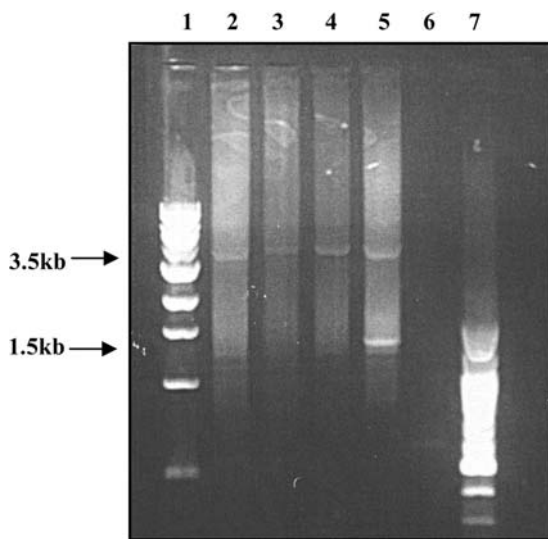
Three individuals having LRH3-GH2.8 autotransgene showed very similar hybridization patterns with two different enzyme-probe combinations. On the other hand, among the five individuals that had LRH3-GHcDNA as transgene integrated into their genome, two individuals (lanes 2 and 3 Fig. 3) showed a similar pattern of hybridization with *HindIII* digestion and three individuals (lanes 1, 4, 6 of Fig. 3) showed a similar pattern of hybridization with *EcoRV* digestion. This similarity was consistent when both GHcDNA (gene) and H3 (promoter) probes (Fig. 3, panel II and III) were used. Similarity in the hybridization patterns in different individuals may indicate that the transgene is inserted at the same site. This raises the question of the presence of recombinational hot spots in these fishes as reported in humans (Callen et al. 1995).

The individuals having LRH3-GH2.8 construct showed strong hybridization signals with both the probes. This may relate to the number of copies of the transgene present in these individuals. Further work needs to be done to confirm this point. The similarity

in the hybridization patterns with GHcDNA and H3 probes reveals the expected co-localization of the bands, which support the intact nature of the integrated transgene (Zhang et al. 1990; Devlin et al. 2004). The observation that the *HindIII* digested sample (lanes 1, 4, 6 in Fig. 3) does not show any hybridization other than with their endogenous genes (H3 and GH), but the same samples digested with *EcoRV* showed clean and strong hybridization signals with both H3 and GH probes indicating the presence of transgene as these individuals were PCR positive. Similar result of PCR positive but Southern negative individuals were reported by Devlin et al. (2004) in transgenic coho salmon.

#### Inverse PCR analysis for the mapping of transgene integration

To determine the integration point of the autotransgene into the genome of the transgenic individuals, inverse PCR (Perucho et al. 1980; Wu et al. 2004) analysis was carried out. DNA from the transgenic individuals after digestion separately with *PstI*, *XbaI*, *NdeI* and *BamHI* were self-ligated and used for PCR amplification with primers LR4EF (5'ATGTTG-AAGCTCCTTCGCATCT, exon 4 of GH in forward orientation) and primer H3R (5'GGCGCTAGC-TAGCTTCCTTCTT-3' histone 3 promoter in reverse orientation). Figure 4 shows the result of the amplification obtained after digestion with *PstI* and after inverse PCR. Amplification is seen in the 3.5 kb region in all transgenic individuals (lanes 2–5 of individual 1, 4, 6 and 8 respectively in Fig. 3). However an additional band in 1.5 kb region is seen in the individual in lane 5, which has transgene, LRH3-GH2.8. These amplified bands were eluted from the gel and subjected to DNA sequencing, the results of which are shown in Fig. 5. Two individuals (individual 1 and 6) with transgene LRH3-GHcDNA were found to have integration into the same fragment of DNA. ClustalX analysis of the sequence shows that the upstream and downstream regions including the transgene are the same in these two individuals. On the other hand, individual 4 having LRH3-GHcDNA and the individual 8 with LRH3-GH2.8 have different sequences both in the upstream and downstream regions of the transgene indicating different integration sites. This study also indicates that the integrated autotransgenes do not show any alteration in



**Fig. 4** Analysis of the GH autotransgene insertion by inverse PCR in *Labeo rohita*. DNA samples from transgenic fishes are digested with *Pst*I and self ligated. PCR is done with primers H3R and LR4EF (Table 1). Lanes 2–4, individuals having autotransgene LRH3-GHcDNA; Lane 5, transgenic individual having autotransgene LRH3-GH2.8 showing additional band at 1.5 kb; Lane 6, non transgenic control. Lane 1 and 7 represent molecular weight markers

individuals 1, 4, 6, and 8, since the sequence of the autotransgene is the same, after inverse PCR amplification (data not shown). The strong hybridization signal in Southern analysis and an additional band at 1.5 kb in inverse PCR of individuals with LRH3GH2.8, may point to the presence of an additional copy of transgene, the origin of which is yet to be determined.

The sequence of the integration site of the transgene when used for BLAST analysis on the fish genome sequences present in the database (Zebrafish, Medaka, Tilapia, Salmon and Trout) did not identify any known gene sequence, whereas Wu et al. (2004) in common carp was able to identify the integration sites of transgenes as being mainly in the regulatory and coding sequences. To identify with certainty the integration site in *L. rohita*, longer stretches of DNA need to be sequenced, which is now in progress. The present study confirms the transgenic nature of different autotransgenic founder *L. rohita* through three independent methods. Work is underway to obtain homozygous autotransgenic *L. rohita* through genetic crossings in fish that has reached sexual maturity.



**Fig. 5** DNA sequence assembly of the inverse PCR product from transgenic individuals. Panel a, 5' end of autotransgene where box indicates H3 promoter region. Panel b, 3' end of

autotransgene where box indicate GH gene. Transgenic fishes are marked by autotransgene construct followed by number

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